Method of modifying peptide synthetases such that they can N-methylate their substrate amino acids

Description

The invention relates to the modification of peptide synthetases (PPS) such that they can N-methylate their substrate amino acids. This is achieved by a specific modification or replacement of the functional subunits (activation domains) of these enzymes.

Peptide synthetases (PPS) are enzymes which synthesize peptides by a non-ribosomal mechanism. The peptides synthesized by the PPS (or derivatives thereof) are often of pharmaceutical interest, e.g. penicillines, vancomycin, cephalosporin, pristinamycin or actinomycin D. The PPS have a modular set-up. Each module of a PPS recognizes, activates and binds one amino acid. Some PPS modules also accept unusual (non-proteinogenic) amino acids as substrates, e.g. alpha-aminoadipinic acid (in penicillin) or phenylglycine (in pristinamycin). The synthesis of a peptide catalyzed by the PPS takes place by the enzyme-catalyzed condensation of the amino acids bound to the modules. This condensation is directed, namely in such a way that the substrate amino acid bound to the first module of the PPS (referred to the N-terminus of the PPS) forms the start (N-terminus) of the synthesized peptide. Thus, the number and order of modules within a PPS determine the length and the sequence of the synthesized peptide (Kleinkauf, H., von Döhren, H. (1990) Eur. J. Biochem. 192:1-15). This is of fundamental importance because the structure of a product obtained after a replacement, insertion or deletion of PPS modules by genetic engineering can be predicted.

All known PPS modules share the feature that they are composed of at least three functional domains (Figure 1A). These three domains are (1) the adenylation domain, necessary for the recognition and adenylation of the substrate amino acid, and (2) the ACP domain, necessary for the covalent binding of the adenylated amino acid as thioester, and (3) the condensation domain, necessary for condensation of all PPS bound amino acids to the synthesized peptide (Stachelhaus *et al.* (1995) FEMS Microbiol. Lett. 125:3-14). Together, the adenylation domain and ACP domain are also described as activation domain (Figure 1A) because together they enable recognition and covalent binding of the substrate amino acid as a reactive thioester. A special group is formed by those activation domains which are also able to N-methylate their substrate amino acids after the covalent binding. With PPS having such activation domains, hence the peptide formed by the subsequent condensation also contains N-methylated amino acids. However, the number of presently known or cloned genes encoding for activation domains with N-methyltransferase activity is substantially lower than the number of activation domains without N-methyltransferase activity (more than 80 domains). Moreover, many of the domains with N-methyltransferase activity have a comparable substrate activity, e.g. for the amino acid in the modules of the actinomycin synthetase II from *Streptomyces chrysomallus* (Schauwecker *et al.* (1998) J. Bacteriol. 180:2468-2474), of the cyclosporine synthetase from *Tolypocladium niveum* (Weber *et al.* (1994) Cur. Genet. 26:120-125) and of the enniatin synthetase from *Fusarium scirpi* (Haese *et al.* (1993) Mol. Microbiol. 7:905-914).

The invention described hereinafter is important because it also allows the conversion of activation domains without N-methyltransferase activity into activation domains with N-methyltransferase activity without altering the original amino acid substrate specificity. Thus, for each specificity of a given PPS module, a corresponding module derivative with additional N-methyltransferase activity can be provided. These derivatives can then be used to construct novel or modified PPS by means of which the peptide synthesized by the PPS is N-methylated at the desired peptide bonds. This allows the synthesis of novel peptides with potentially new pharmacological properties. Many of the already known pharmacologically active peptides or peptide derivatives contain N-methylated amino acids, e.g. cyclosporine. In contrast to the invention, selective N-methylation of particular nitrogen atoms within the peptide bonds of polypeptides is only hard to accomplish or can even not be achieved by chemical methods.

The invention is based on the finding that all activation domains with N-methyltransferase activity harbor an additional domain which is localized between the adenylation and ACP domain (Figure 1B). This additional domain, designated N-methyltransferase domain hereinafter, mediates N-methylation of the bound substrate amino acid. The invention comprises methods for the conversion of activation domains without N-methyltransferase activity into activation domains with N-methyltransferase activity and the use thereof for reconstructing PPS for the synthesis of N-methylated amino acids and peptides. There are two basic approaches by which activation domains without N-methyltransferase activity of a PPS can be converted into activation domains with N-methyltransferase activity:

(1) Replacing a complete module or the complete activation domain of a PPS. This method is described in Example 2.

(2) Inserting a N-methyltransferase domain as a functional unit into an activation domain. For example, the N-methyltransferase domain can directly be inserted between the adenylation domain and ACP domain of the activation domain which is to be converted (Figure 2A). Two adjacent fusion sites can also be used for the insertion. In this case, the part between those fusion sites of the activation domain which is to be converted will be deleted and replaced by corresponding parts which will be inserted together with the N-methyltransferase domain (Figure 2B). This method is described in Example 3. If two fusion sites are used, the N-methyltransferase domain can also be inserted after the activation domain as an elongated unit with a tailing ACP domain (or parts thereof) leading to the replacement of the original ACP domain by the inserted ACP domain (or parts thereof) (Figure 2C and 2D). However, the substrate specificity of the converted activation domain is retained for each of the insertion approaches since the insertion does not alter the adenylation domain (recognition and adenylation of the substrate amino acid).

Suitable insertion sites for inserting a N-methyltransferase domain into an activation domain are determined by the transition between the adenylation domain and ACP domain. These result from the sequence comparison between activation domains with N-methyltransferase domain and activation domains without N-methyltransferase domain (Figure 3). The N-methyltransferase domains are located as insertions about 45 amino acids after (C-terminal) the adenylation domain consensus sequence QVKIRG(F/H/Y)RIE(L/I)GEIE, known as "core motif 5" (Turgay *et al.* (1992) Mol. Microbiol. 6:529-546),

and immediately N-terminal to the consensus sequence (Q/E/D)(I/V)REx(V/L)xxxLPXYM(V/I)P.

All of the above described methods for converting an activation domain without N-methyltransferase activity into an activation domains with N-methyltransferase activity or the use thereof for constructing novel PPS comprise a specific alteration and combination of the corresponding DNA regions of peptide synthetase genes. This is done by inserting the DNA region, which encodes for the N-methyltransferase domain of any activation domain with N-methyltransferase activity, into the DNA segment encoding for the activation domain which is to be converted. The DNA must to be inserted such that a continuous reading frame is obtained after insertion and that the encoded N-methyltransferase domain becomes an integral part of the encoded activation domain. For this, the DNA fragment of a PPS gene for example, which completely or partially encodes for the activation domain which is to be converted, or parts thereof, may be cloned in plasmids. All standard techniques of molecular biology, e.g. the polymerase chain reaction (PCR) may be used for cloning and modifying of DNA. Cloning and DNA manipulations may be carried out in all plasmids and organisms suitable for these purposes, e.g. pUC plasmids and *E. coli*. Restriction sites may be used for cloning and modifying of DNA which are already present or which may be generated by PCR for example. Such methods are described in Example 1 and comprise the introduction of a restriction site into the actinomycin synthetase II gene which is used for the subsequent module replacement.

New PPS genes can be constructed by inserting a DNA fragment encoding for the N-methyltransferase domain into a PPS gene

segment. Expression of a novel PPS gene can be carried out using plasmids and may result in the synthesis of new products. This is described in Example 4 and comprises the expression of a recombinant PPS gene after a corresponding plasmid has been transformed into *Streptomyces lividans* and the verification of the catalytic activity of the PPS encoded by the PPS gene. DNA fragments may also be used to introduce PPS genes into the genome of organisms or to modify PPS genes already present in the genome as it was shown for example for the surfactin synthetase gene of *Bacillus subtilis* (Stachelhaus *et al.* (1995) Science 269(5220):69-72). Therefore, modules with N-methyltransferase activity can also be introduced into genomic PPS genes and which may result in the formation of novel, N-methylated peptides.

Examples

The method according to the present invention is described with the help of Examples in more detail hereinafter.

The plasmids used for the realization of the Examples (pSP72, pBlueScript, pIJ702, pSPIJ004 and pACM5) are schematically shown in Figure 4 and further explained in Table 1.

DNA sequences of the oligonucleotides used for PCR are listed in Table 2. Sizes of PCR fragments as given in the Examples correspond to PCR fragments which have been obtained after digestion with the restriction enzymes indicated in the Examples. Additional restriction sites in the oligonucleotides were used to clone the PCR fragments into *E. coli* standard plasmids first before carrying on with the cloning steps described as in the Examples.

The DNA sequence of the actinomycin synthetase II gene (*acmB*) has the "GenBank" data base entry AF047717. The DNA sequence of a 3849 bp *Bam*HI fragment, derived from the actinomycin synthetase III gene (*acmC*), is attached to the examples hereinafter.

Example 1 Introduction of a restriction site into the actinomycin synthetase II gene in order to enable the replacement of an activation domain.

The actinomycin synthetase II (ACMS II) from *Streptomyces chrysomallus* possesses two modules without N-methyltransferase activity of which module 1 and 2 are activating threonine and valine, respectively. In order to be able to replace the activation domain of module 2, a *Eco*RV restriction site was introduced by mutagenesis into the ACMS II gene (*acmB*). This *Eco*RV restriction site and a *Cla*I restriction site which are already present in the gene allow to replace the region which encodes for the activation domain of module 2 by any given *Cla*I-*Eco*RV fragment. The replacement comprises numerous cloning steps which will be formally described first and thereafter in more detail.

1. Formal summary of the cloning strategy

Plasmid pACM5 was used to generate a *Eco*RV restriction site within the ACMS II gene (*acmB*) (Figure 4; Schauwecker *et al.* (1998) J. Bacteriol., 180:2468-2474). Plasmid pACM5 (Figure 4) harbors the gene *acmB* following a constitutive Streptomyces promotor (*melP*) and is a derivative of the Streptomyces plasmid pIJ702. An *Eco*RV restriction site was introduced by PCR mutagenesis and corresponding cloning steps into the gene *acmB* after the phosphopantetheine binding site

encoding region (in module 2) at base pair (bp) position (pos.) 6251.

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                                          V  R  D  V  F  E
acmB wildtype      (bp 6244-6262) :  5'- gtccgggacgtcttcgag
                                          (bp pos. 6251)

                                          V  R  D  I  F  E
acmB mutagenized   (bp 6244-6262) :  5'- gtccgggatatcttcgag
                                           EcoRV
                                          (bp pos. 6251)
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2. Detailed description of the individual cloning steps

First, a 4923 bp *Pst*I-*Cla*I fragment, comprising the *mel* promotor and most of the 5'-located region of *acmB* (down to the *Cla*I restriction site at bp pos. 4519 in *acmB*), was isolated from pACM5 and cloned into *E. coli* plasmid pSP72 (A in Figure 5). Then, part of the adjacent 3'-region of *acmB* (starting from the *Cla*I restriction site at bp pos. 4519) was amplified by PCR using the oligonucleotides *prim*-A and *prim*-B (PCR fragment 1 in Figure 5) and was inserted as 1737 bp *Cla*I-*Eco*RV fragment (B in Figure 5). Primer *prim*-B introduces an *Eco*RV restriction site corresponding to bp pos. 6251 in *acmB*. The assembled fragments were then isolated as a complete *Pst*I-*Eco*RV fragment and cloned into pBlueScript (C in Figure 5). The assembled 5'-region of *acmB* can then be isolated as *Bam*HI-*Eco*RV fragment therefrom for subsequent cloning. The still missing 3'-region of *acmB* was amplified using primer *prim*-C and *prim*-D (PCR fragment 2 in Figure 5) and was cloned as 2583 bp *Eco*RV-*Bam*HI fragment into pSP72 (D in Figure 5). The resultant plasmid was digested with *Bgl*II and *Eco*RV and the 5'-region of *acmB* (isolated as *Bam*HI-*Eco*RV fragment as described above) was inserted. This results in plasmid pACM00-A (Figure 5) which harbors the completely

assembled gene *acmB* having an *Eco*RV restriction site introduced at bp pos. 6251.

Example 2 Replacement of a complete activation domain without N-methyltransferase activity by a activation domain with N-methyltransferase activity within a PPS.

The replacement of a complete activation domain was performed within the actinomycin synthetase II (ACMS II) from *Streptomyces chrysomallus*. The activation domain of module 2 was replaced by an activation domain with N-methyltransferase activity. The activation domain with N-methyltransferase activity which was used for the replacement was derived from the actinomycin synthetase III (ACMS III) and is equally specific for valine. The replacement comprises numerous cloning steps which will be formally described first and then in more detail.

1. Formal summary of the cloning strategy

The region between a *Cla*I restriction site in *acmB* at bp pos. 4519 and an *Eco*RV restriction site introduced at bp pos. 6251 (in plasmid pACM00-A from Example 1), which is encoding for the second activation domain of ACMS II, was deleted and replaced by a PCR generated 2961 bp *Cla*I-*Eco*RV fragment, which is encoding for an ACMS III activation domain with N-methyltransferase activity having specificity for valine. The regions at the fusion sites (*Cla*I and *Eco*RV) encode for segments which are conserved in both PPS and located N- and C-terminal towards the activation domains. Insertion of the PCR generated *Cla*I-*Eco*RI fragment into the modified gene *acmB* results again in a continuous reading frame encoding a recombinant ACMS II.

modified	S R I D V L T	S V R D I F E
ACMS II	.. agccgtatcgatgtcctcacc.....	.....tccgtccgggacgtcttcgag ..
(in plasmid pACM00-A)	*ClaI*	*EcoRV*
	(bp pos. 4591)	(bp pos. 6251)

activation domain	V L T	G L R . .
of ACMS III	atcgatGTCCTCACC.....	.....GGCCTGCGCgatatc
(2961 bp PCR-fragment)	*ClaI*	*EcoRV*

recombinant	S R I D V L T	G L R D I F E
ACMS II	.. agccgtatcgatGTCCTCACC.....	.....GGCCTGCGCgatatcttcgag ..
(in plasmid pACM00-B)	*ClaI*	*EcoRV*
	(bp pos. 4591)	(bp pos. 7480)

The gene of the recombinant ACMS II (in plasmid pACM00-B, Figure 7) was transformed into *Streptomyces lividans* and the catalytic activity of the introduced activation domain was verified after expression of the PPS gene as described in Example 4.

2. Detailed description of the individual cloning steps

A 2967 bp *Cla*I-*Eco*RV fragment of a 3849 bp *Bam*HI fragment derived from the ACMS III gene (*acmC*, sequence is attached), which encodes for a valine activation domain with N-methyltransferase activity, was amplified by PCR using the oligonucleotides *prim*-E and *prim*-F (PCR fragment 4 in Figure 6). This *Cla*I-*Eco*RV fragment was cloned into the plasmid pACM00-A (from Example 1), whereby the *Cla*I-*Eco*RV fragment originally present in pACM00-A was replaced. The resulting plasmid was digested with *Bam*HI and *Hind*III and the Streptomyces part from pSPIJ004 (Figure 4) was inserted as a 5130 bp *Bgl*II-*Hind*III fragment. This generates the plasmid pACM00-B (Figure 7) which can be transformed and selected in both *E. coli* and Streptomyces.

Example 3 Conversion of an activation domain without N-methyltransferase activity into an activation domain with N-methyltransferase activity and introducing said converted activation domain into a PPS.

An additional N-methyltransferase domain was inserted into the valine activation domain of module 2 of ACMS II between the adenylation domain and the ACP domain. Thereby, the activation domain of ACMS II is provided with an additional N-methyltransferase activity. The inserted N-methyltransferase domain is derived from module 3 of the ACMS III. The replacement comprises numerous cloning steps which will be formally described first and thereafter in more detail.

1. Formal summary of the cloning strategy

First, two *Sna*BI restriction sites were introduced by PCR mutagenesis at bp pos. 5899 and bp pos. 5932 in gene *acmB* for the intended insertion of a N-methyltransferase domain. The region of 33 bp length between the two *Sna*BI restriction sites was then deleted and replaced by an 1263 bp *Eco*RV-*Eco*RV fragment encoding the above-mentioned N-methyltransferase domain of ACMS III. The ligation of the *Sna*BI ends with the *Eco*RV ends results in the formation of a DNA sequence which is no longer cleavable by both restriction enzymes. A new reading frame, encoding for a recombinant ACMS II, is obtained after inserting the *Eco*RV-*Eco*RV fragment for one of the possible two orientations.

```
                                R L V A Y V V A D G G T A P D G L R E A L
ACMS II                 .. cgcctcgtcgcctacgtcgtcgcggacggcggaacggccccggacggtctgcgcgaggccctc ..
                                        (bp pos. 5899)                  (bp pos. 5932)

modified                        R L V A Y                                       V R E A L
ACMS II                 .. cgcctcgtcgcctacgtagtcgcggacggcggaacggccccggactacgtacgcgaggccctc ...
                                        SnaBI                                   SnaBI
                                        (bp pos. 5899)                  (bp pos. 5932)

N-methyltransferase domain                      I V A D                 L L T D
of ACMS III                             gatATCGTCGCGGAC.........CTGCTCACCGATatc
(1263 bp PCR-fragment)                  EcoRV                           EcoRV

recombinant                     R L V A Y I V A D                L L T D V R E A L
ACMS II                 .. cgcctcgtcgcctacATCGTCGCGGAC.........CTGCTCACCGATgtacgcgaggccctc ..
(in plasmid pACM00-C)                   (bp pos. 5899)                  (bp pos. 7156)
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The gene of the recombinant ACMS II (in plasmid pACM00-C, Figure 7) was transformed into *Streptomyces lividans* and the newly introduced N-methyltransferase activity of the recombinant PPS was verified as described in Example 4.

2. Detailed description of the individual cloning steps

In order to introduce the *Sna*BI restriction sites, the region of the gene *acmB* from bp pos. 4591 to 5899 as well as the region from bp pos. 5932 to 6251 were amplified by PCR using the oligonucleotides *prim*-G and *prim*-H (PCR fragment 1 in Figure 6) and *prim*-I and *prim*-J (PCR fragment 2 in Figure 6), respectively. Thereafter, the PCR fragment 2 was cloned as 330 bp *Hind*III-*Eco*RV fragment into pBlueScript first and the PCR fragment 1 was then inserted as 1386 bp *Cla*I-*Sna*BI fragment. This results in a DNA fragment which encodes for the almost complete activation domain of module 2 of the ACMS II and in which a *Sna*BI restriction site was introduced (A in

Figure 6). A 1263 bp *Eco*RV-*Eco*RV fragment (PCR fragment 3 in Figure 6), which was amplified from a 3849 bp *Bam*HI fragment derived from the ACMS III gene (*acmC*, sequence is attached) by PCR using the oligonucleotides *prim*-K and *prim*-L, was then inserted in that *Sna*BI restriction site. The orientation of the inserted *Eco*RV-*Eco*RV fragment which is encoding for the N-methyltransferase domain of ACMS III was verified by DNA sequencing. Because of the fusion of the *Eco*RV ends with the *Sna*BI ends, the assembled activation domain could then completely be isolated as a 2961 bp *Cla*I-*Eco*RV fragment which was cloned into plasmid pACM00-A (from Example 1) and thereby, the *Cla*I-*Eco*RV fragment originally present in pACM00-A was replaced. The resulting plasmid was digested with *Bam*HI and *Hind*III and the Streptomyces part derived from plasmid pSPIJ004 (Figure 4) was inserted as a 5130 bp *Bgl*II-*Hind*III fragment. This generates the plasmid pACM00-C (Figure 7) which can be transformed and selected in both *E. coli* and *Streptomyces*.

Example 4 Expression of recombinant PPS with introduced N-methyltransferase domain and *in vitro* analysis of their N-methyltransferase activity.

For the expression of the PPS genes which were constructed according to Examples 2 and 3, the plasmids pACM00-B and pACM00-C (Figure 7), which are described there, were transformed into *Streptomyces lividans* (strain TK64). Transformation as well as microbiological cultivation of Streptomyces were performed according to standard protocols (Hopwood *et al.* (1985) Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, England). Plasmid-encoded PPS were purified from stationary growing transformants (after 3-days-growth) obtained from 1

liter of YEME. The purification of PPS up to a stage necessary for enzymatic analysis is essentially based on a protocol previously described in detail (Schauwecker *et al.* (1998) J. Bacteriol. 180:2468-2474) and is therefore described only schematically:

Proteins were released from cells by mechanic cell disrupture (French press). Simultaneously released genomic DNA was digested with DNAseI to obtain a fluid suspension. Cell fragments were removed by centrifugation and proteins were then precipitated by addition of ammonium sulfate up to a final concentration of 55%. Precipitated proteins were size-fractionated by exclusion chromatography (column matrix: Ultrogel-AcA-34 from Biosepra). Protein fractions with protein having a size larger than 200 kDa were pooled and further purified on an anion exchanger (column matrix: Q-Sepharose FF from Pharmacia). Proteins bound on the anion exchanger were release from the anion exchanger by continuously adding NaCl. The PPS which were constructed according to Examples 2 and 3 eluted in a range between 150 to 250 mM NaCl. PPS partially purified according to this protocol can then be further analyzed, for example according to the protocols as given below:

<u>Example protocol as to how to verify the specific recognition and binding of amino acids by a PPS *in vitro*:</u>

Mix 100 µl of a partially purified PPS with 3 µl of ^{14}C-labelled substrate amino acid (100 µCi/ml), 2 µl $MgCl_2$ (1 M) and 15 µl ATP (0.1 M) and incubate for 30 minutes at 30 °C. Precipitate the PPS by adding 2 ml of 7% trichloracetic acid (TCA), wash with 10 ml 5% TCA and quantify the amount of substrate amino acid bound to the enzyme by measuring the radioactivity.

Example protocol as to how to verify the N-methylation of substrate amino acids catalyzed by a PPS *in vitro*:

To verify the N-methylation activity, incubate the PPS with ^{14}C-labelled substrate amino as described above but complement the incubation mix by adding 3 µl of 0.1 M S-adenosyl methionine (SAM) as a donor of the methyl group which is to be transferred to the amino acid. After TCA precipitation, wash the PPS with 4 ml of 5% TCA (two portions), then wash with 2 ml ethanol and dry at 37 °C. Add 300 µl performic acid and incubate for 6 hours at 20 °C to release the substrate amino acid bound as thioester. Then vacuum dry the mixture. Dissolve the amino acid by adding 40 µl formic acid and verify the conversion into the N-methylated form, e.g. by chromatographic methods. For example, the conversion of valine into N-methyl valine can be shown as follows: Chromatograph 20 µl of the (^{14}C-labelled) amino acid released from the PPS in parallel to 5 µl of the corresponding references (0.1 M valine and 0.5 M N-methyl valine) on a silica 60 thin-layer chromatography plate (Merck) using the solvent system n-butanol : acetic acid : water (volume 80:20:20). Visualize the amino acids by a ninhydrin reaction and a autoradiogram for the ^{14}C-labelled amino acid.

Example protocol as to how to verify the formation of peptides catalyzed by a PPS *in vitro*:

In general, a peptide can simply be analyzed by acidic hydrolysis followed by the determination of the individual amino acid components. This applies especially to peptides which are formed by PPS since the amino acid sequence of the

synthesized peptide is already known from the module arrangement. Because of the use of ^{14}C-labelled amino acids, the analysis of the *in vitro* formed peptide can be performed as follows: Incubate 100 µl of partially purified PPS with each of the PPS substrate amino acids (2 mM each), SAM (2 mM), ATP (10 mM) and $MgCl_2$ (20 mM) in a total volume of 150 µl for 25 minutes at 30 °C. If necessary, the mixture may contain further enzymes which are co-acting with the PPS which is intended to be analyzed (Pfennig *et al.* (1999) JBC 274:12508:12515). Prepare more than one incubation mixture in parallel, in which the number of the incubation mixtures is dependent on the number of modules within the PPS and use the ^{14}C-labelled amino acid which corresponds to the module in each of the incubation mixtures. Precipitate the PPS in each of the incubation mixtures with TCA as described above, cleave off the synthesized peptide with performic acid, dry and dissolve the formed peptide in ethanol / water (volume 1:1) and verify the peptide by chromatographic methods. For example, to verify the threonyl-N-methyl-valine peptide linkage by the PPS constructed according to Examples 2 and 3 one can proceed as follows: Chromatograph 20 µl of the peptide released from the PPS of each incubation mixture (one with ^{14}C-labelled threonine and one with ^{14}C-labelled valine) on a silica 60 thin-layer chromatography plate (Merck) using the solvent system n-butanol / acetic acid / water (volume 80:20:20). Isolate all products formed in both incubation mixtures having an identical R_f value by extraction using ethanol / water (volume 1:1), vacuum dry and release the amino acids from the peptides by acidic hydrolysis (6 N HCl, 110 °C, 20h). The identification of the released ^{14}C-labelled amino acids is again performed by chromatography on thin-layer chromatography plates using the same solvent system. This allows to identify the components threonine and N-

methyl-valine in the formed peptide. Furthermore, a peptide reference can directly be compared with the enzymatically formed and ^{14}C-labelled peptide, e.g. by HPLC using a column designed for peptide separation like the SuperPac Pep-5 column from Pharmacia, if synthesis of the reference peptide by chemical means is possible.

Tables and Figures

Table 1

Starting plasmids used for realizing the Examples

plasmid	origin or literature quotation	selec-tion	description
pSP72	Promega	Amp	commercial cloning vector for *E. coli*
pBlue-Script	Stratagene	Amp	commercial cloning vector for *E. coli*
pIJ702	Katz *et al.* (1983) J. Gen. Microbiol. 129 : 2703-2714	Tsr	Commonly used cloning vector for Streptomyces. It harbours the melanin (mel) genes *melC1* and *melC2* under control of their promotor (mel P).
pSPIJ004	own development	Amp Tsr	The plasmid is a combination of pSP72 and pIJ702 and is replicable both in *E. coli* and in Streptomyces. For this purpose, the *PstI-BglII* fragment from pIJ702 was cloned into the polylinker of pSP72.
pACM5	Schauwecker *et al.* (1998) J. Bacteriol. 180 : 2468-2474	Tsr	The plasmid is a pIJ702 derivative and harbours the actinomycin synthetase II gene (*acmB*) under control of the *mel*-promotor.

abbreviations: Tsr = thiostreptone, Amp = ampicillin

Table 2

PCR oligonucleotides used in the Examples

oligonucleotide	DNA sequence and restriction sites
prim - A	5´- gccggaattccgtatcgatgtcctcaccccggaggaga *EcoRI* *ClaI*
prim - B	5´- tgcggaattcgaagatatcccggacggagaaaccgat *EcoRI* *EcoRV*
prim - C	5´- tctccgtccgggatatcttcgagcagcgcacg *EcoRV*
prim - D	5´- atggcctgagttgctggatcctggcgatcccga *BamHI*
prim - E	5´- ctcagccgcatcgatgtcctca *ClaI*
prim - F	5´- cgcctcgaagatatcgcgcaggccca *EcoRV*
prim - G	5´- gcaggaattcagccgtatcgatgtcctca *EcoRI* *ClaI*
prim - H	5´- ttccggaattcgcgactacgtaggcgacga *EcoRI* *SnaBI*
prim - I	5´- cggccaagctttacgtacgcgaggccctccggcggcgcct *HindIII* *SnaBI*
prim - J	5´- tgcggaattcgaagatatcccggacggagaaaccgat *EcoRI* *EcoRV*

Nucleotide sequence of the *BamHI* fragment from the gene *acmC* used for realizing the Examples

nucleotide sequence:	numbering of base pairs
GGATCCACCT GCTCGACACC GCCACCGCCC AACCCGAGCA GCCCCTCAGC CGCATCGACG	0000000060
TCCTCACCCC GGAGGAGAGG AACCGCACGA TCGTCGAGGT CAACCGGACC GAACTGCCGC	0000000120
TGCCCGACGC CTCGTTGGCG GAGCTGTTCG AACAACAGGT GACCCTCACA CCCGACGCCC	0000000180
CCGCCCTGGT CAGCGACGGC GCCACGCTCA GCTACTCCGA GCTCAACACG CGCGCCAACC	0000000240
ACCTCGCCCA CCAGCTCACC ACCCGGGGCA TCCGCCCCGG CGACGCCGTC GCCGTCCTCC	0000000300
TCCAACGCTC CCCCGACACC GTCACCACCG TCCTCGCCCT CGCCAAGACC GGCGCGACCT	0000000360
ACATCCCCCT CGACAGCCGC TACCCCGCCG ACCGCTACCG CCTCGTCCTC GACGAGACCC	0000000420
GCACCAAACT CCTCATCACC GACCACACCA CCGACCTCGA CACCACCACA ACCCAGTTCA	0000000480
ACCCCGCCGA CACCCCCCAC GACGGCGAAG ACCCCGGCAA CCCGAACCAC ACCACCCACC	0000000540
CCGACGACGC CGCCTACATC ATGTACACCA GCGGCTCCAC CGGCCGCCCC AAGGGCGTCA	0000000600
TCGCCACCCA CCGCAACATC ACCGCCCTCG CCCTCGACCC CCGCTTCGAC CCCACCGCCC	0000000660
ACCGCCGCGT CCTCCTCCAC TCCCCCACCG CCTTCGACGC CTCCACCTAC GAGATCTGGG	0000000720
TCCCCCTCCT CAACGGCAAC ACCGTCGTCC TCGCCCCCAC CGGCGACCTC GACGTCCACA	0000000780
CCTACCACCG CGTCATCACC GACCAGCAGA TCACCGCCCT CTGGCTGACC AGCTGGGTCT	0000000840
TCAACCTCCT CACCGAGCAG AGCCCGGAGA CCTTCACCCG GGTCCGGCAG ATCTGGACCG	0000000900
GCGGCGAGGC CGTCTCCGGC GCCACCGTCA CCCGGCTTCA GCAGGCATGC CCCGACACCA	0000000960
CCGTGGTCGA CGGCTACGGC CCCACCGAGA CCACCACCTT CGCCACCCAC CACCCCGTCC	0000001020
CCACCCCCTA CACCGGCTCC GCCGTCGTCC CCATCGGCCG CCCCATGGCC ACCATGCACA	0000001080
CCTACGTGCT CGACGACAGC CTCCAGCCCG TCGCCCCCGG CGTCACCGGC GAGCTCTACC	0000001140
TCGCTGGCAG CGGCCTCGCC CGCGGCTACC TGGACCGCCC CGCCCTCACC GCCGAACGCT	0000001200
TCGTCGCCAA CCCGTACGCC GCACCCGGAG AACGCATGTA CCGCACCGGC GACCTGGCAC	0000001260
GCTGGAACCC CGACGACCAC CTCGAGTACG CCGGCCGCGC CGACCACCAG GTCAAGGTCC	0000001320
GCGGCTTCCG CATCGAACCC GGCGAGATCG AGAACGTCCT CACCGACCAT CCCGCCGTCG	0000001380
CCCAGGCCGC CGTCCACCTC AACCGGGACC AGCCCGGCAA CCCCCGGCTC GTCGCGTACG	0000001440
TCGTCGCGGA CACCTCGGCG CCGAGCAGCG ATGTGGACCA GCAGCACCAG ATCGGCGAGT	0000001500
GGCAGGACCT CTACGACTCC CTCTACGCGG CCCCCACGGC CGAGTTCGGC GAGGACTTCT	0000001560
CCGGCTGGAA CAGCAGCTAC GACGGCCGGC CGATCCCCCT CGACCAGATG CGGGAGTGGC	0000001620
GCGACGCCAC CGTGGAACGC ATCCGCGGCC TCAACCCGCG CCGGGTGCTG GAGATCGGCG	0000001680
TCGGCACGGG CCTGCTGCTC GCGAAGCTGG CCCCCGAGTG CGAGGAGTAC TGGGGCACGG	0000001740
ACCTCTCGCC CACCGTGATC GAGGCGCTCT CCCGGCACGT CGACGCCGAC CCGGAGCTGG	0000001800
CCCGGCGGGT CACCCTGCGG GCCGGTGCCG CGCACGAGCA CGAGGGGCTG CCCGTCGGCC	0000001860
ACTTCGACAC CGTCGTGCTC AACTCCGTGG TCCAGTACTT CCCGAACGCC GACTACCTCG	0000001920
CCCAGGTCAT CGAGCAGGCG CTGCGGCTGC TGGCCCCCGG CGGCGCCGTG TTCATCGGCG	0000001980
ACATCCGCAA CCCGCGGCTG CTGCGCACCT TCACCACCGC CGTCCAGACC GCCCGCGCGG	0000002040
AGGACCCGGC CGACACCGCC GCCGTGCGGC GCGCCGTCGA GCAGAGCCTG GTGCTGGAGA	0000002100
AGGAACTCCT GGTCGACCCG GAGTACTTCA CCGCGCTCAC CCACCGCCTC CCGGACCTCG	0000002160
CCGGCGTCGA CCTGCGGCTC AAGTGCGGCG CCGCCCACAA CGAGTTGACC CGCTACCGCT	0000002220
ACGACACCAC GCTCCACAAG GCCGGAATCA CCGCGCTCCC GCTGTCCGAG GCCGCCGTCC	0000002280
TGGCCTGGCC GCAGGACGCC GAGGCACTCG CCCGGCACCT GGCCGAGGCC CGGCCGGAGC	0000002340
GGCTGCGCGT CACCGGCGCG CCCAACTCCC GGATAGCCGC CGACCTCGCG GCCCAGCACG	0000002400
CCCTGGAGTC CGGCACCGCC CCGGCCGGGC CCCCGACCGG GCCCTACGCC ACGGAGCAGC	0000002460
CGGACCTCGA GGCACTCCAC CGCCTCGGGG AGGACCACGG GTACTGGACG GCCGTCACCT	0000002520
GGTCCGCCCA CCGCCCCGAC ACCGTCGACC TCACCTTCGT CCGGCGCGGC CTGCTCGACG	0000002580
GCGCCGTCCC GGTCGGTACG TACGCCCCGG CGGCCGCCGG CGACCCGGCG ACGCCGCTCA	0000002640

CCGCCTTCAC CACCAACCCC GTCGGCAGCC GGGGCACCGC CGCGCTGCTC ACCGCGCTGC 0000002700
GCGAACACGC CGCCGCCCAA CTGCCCGACT ACATGCGGCC CGCCGCAATC GTCCCGCTCG 0000002760
ACCGCCTGCC GCTCACCGCC AACGGCAAGC TCGACCGGGC CGCCCTCCCG GCACTCGACC 0000002820
CGGAGCACGC GGACACCGGC CGCGCCCCCA GGACGCCGCA GGAGCAGGTG GTCTGCGAGC 0000002880
TGTTCGCGGA GGTGCTCGGC CGGCCGCTCG TCGGTGTGGA CCAGGACTTC TTCGACCTCG 0000002940
GCGGGCACTC GCTGCTCGCC ACCCGGCTGA TCGCCCGGCT GCGCGCCGCC TTCGGCGTGG 0000003000
AACTGGGCCT GCGCAGCCTC TTCGAGGCGC CGACGCCGGG CGGGATCGCC GCCCGGCTGG 0000003060
ACCTCGACGA CCCGGACGGC TCCTACGAGG TGGTGCTGCC GCTGCGCGCC CAGGGCAGCA 0000003120
GGCCGCCGCT GTTCTGCATC CACCCCGGTG GCGGCATCAG CTGGTCGTAC AGCGCGCTGA 0000003180
TCAAGCACCT CGGCCCGGAG TACCCGCTGT ACGGCATCCA GGCGCGCAGC CTGGCCCGCC 0000003240
CGGAGCCGCG GCCGGAGAGC ATCGAGGAGA TGGCGGTGGA CTACGCCGAC CAGATCCAGG 0000003300
GCGTGCAGCC GCACGGCCCC TACCACCTGG CCGGCTGGTC GTTCGGCGGG CTGTGCGCCC 0000003360
ATGCCCTGGC CGCGGAGTTC CAGCGGCGCG GCGAGCCGGT GGCGCTGGTC GCGGTGCTCG 0000003420
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TGATGCTGCT GTACCACGTC GGCCTGGTCG ACGACGGCAG CCACCGCAAC GACCGCGAAG 0000003540
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TCGACTACCA GCCCGGCCCG ATCGACGGCG ACCTGCTGCT GATCGCCGCC TCGGAACAGC 0000003720
AGGACCCGCC GGTCACCGCC GATGCCTGGC GGCCGTACGT CTGCGGCGCG GTCGAGGCCC 0000003780
ACGTGGTGCC CGGCGAGCAC GGCTCCATGC TGACCCGGCC CGGCACCCTG GCCGAGATCG 0000003840
GCCGGATCC 0000003849

Figure 1: shows the schematic modular set-up of PPS and the subdivision in functional domains.

Figure 2: shows the modification of activation domains by insertion of a N-methyltransferase domain.

Figure 3: shows the sequence comparison of selected activation domains in the transition regions towards the N-methyltransferase domains.

Figure 4: shows the starting plasmids used in the Examples.

Figure 5: shows the introduction of an *Eco*RV restriction site into *acmB*.

Figure 6: shows the cloning of *Cla*I-*Eco*RV cassettes for the construction of recombinant *acmB* genes.

Figure 7: shows plasmids for the expression of recombinant PPS genes.